Cyclic AMP Immunoassay

Catalog Number DE0450

For the quantitative determination of Cyclic AMP (cAMP) concentrations in tissue, cell lysates, cell culture supernates, saliva, serum and plasma.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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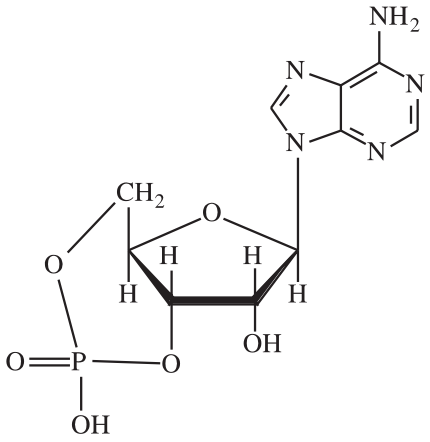
INTRODUCTION

Adenosine 3′, 5′-cyclic monophosphate (cyclic AMP, cAMP) is one of the most important "second messengers", involved as a modulator of physiological processes (1 - 5). cAMP is also involved in regulating neuronal, glandular, cardiovascular, immune, and other functions and actions (6 - 9). A number of hormones are known to activate cAMP through the action of the enzyme adenylate cyclase which converts ATP to cAMP. These hormones include a variety of anterior pituitary peptide hormones such as corticotropin (ACTH), glucagon, calcitonin, thyroid stimulating hormone (TSH) and luteinizing hormone (LH). cAMP has been shown to be involved in the cardiovascular, nervous system, and immune mechanisms, cell growth and differentiation, and general metabolism (10 - 12). There remains considerable interest in the measurement of intracellular cAMP in tissues and cell cultures, and this may help to provide an understanding of human physiology.

cAMP



R&D Systems' cAMP Immunoassay is a 3 hour competitive enzyme immunoassay designed to measure cAMP in tissue, cell lysates, cell culture supernates, saliva, serum and plasma.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique in which cAMP present in a sample competes with a fixed amount of alkaline phosphatase-labeled cAMP for sites on a rabbit polyclonal antibody. During the incubation, the polyclonal antibody becomes bound to the goat anti-rabbit antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The color development is stopped and the absorbance is read at 405 nm. The intensity of the color is inversely proportional to the concentration of cAMP in the sample.

TECHNICAL HINTS

- **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix reagents from different lots.
- Allow reagents to warm to room temperature before use.
- Care must be taken to avoid contamination with endogenous alkaline phosphatase. The unopened bottle of pNPP Substrate should be colorless to pale yellow. Contaminating alkaline phosphatase may lead to high blanks. Care should be taken to avoid touching pipette tips and other items that are used in the assay with bare hands.
- Stop Solution should be added to the plate in the same order as the pNPP Substrate. The color developed in the wells will remain yellow upon the addition of Stop Solution.
- When mixing protein solutions, always avoid foaming.
- Pre-rinse the pipette tip when pipetting standards.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Pipette standards and samples to the bottom of the wells.
- Add all other reagents to the side of the wells to avoid contamination.
- If samples generate values higher than the highest standard, further dilute the samples with Assay Buffer ED2 and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Alkaline phosphatase is a temperature sensitive enzyme. Optical Density (O.D.) units may vary with temperature changes.

REAGENTS

Microplate (Part R80-0060) - 96 well microplate coated with a goat anti-rabbit polyclonal antibody.

cAMP Conjugate (Part R80-0053) - 6 mL of cAMP conjugated to alkaline phosphatase, with blue dye and preservative.

cAMP Standard (Part R80-0056) - 0.5 mL of cAMP (2000 pmol/mL) in buffer, with preservative.

cAMP Antibody Solution (Part R80-0604) - 6 mL of rabbit polyclonal antibody to cAMP, with yellow dye and preservative.

Assay Buffer ED2 (Part R80-0055) - 30 mL of a buffered protein base with preservative.

Wash Buffer Concentrate (Part R80-0016) - 30 mL of a 10-fold concentrated solution of a buffered surfactant with preservative.

pNPP Substrate (Part R80-0075) - 20 mL of p-nitrophenyl phosphate in a buffered solution.

Stop Solution (Part R80-0247) - 5 mL of a trisodium phosphate (TSP) solution. **Keep tightly capped. Caution: caustic.**

Plate Cover - 1 adhesive strip.

Triethylamine (Part R80-0063) - 2 mL of Triethylamine. **Caution: flammable, corrosive, lachrymator.** See Material Safety Data Sheet.

Acetic Anhydride (Part R80-0064) - 1 mL of Acetic Anhydride. **Caution: flammable, harmful, lachrymator.** See Material Safety Data Sheet.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened Reagents	Diluted Wash Buffer	May be stored at room temperature for up to 3 months.*
	Stop Solution	May be stored at 2 - 8° C until the expiration date of the kit.
	Assay Buffer ED2	
	pNPP Substrate	
	Conjugate	
	Antibody Solution	
	Standard	
	Triethylamine	
	Acetic Anhydride	
	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored at 2 - 8° C until the expiration date of the kit.

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 405 nm with wavelength correction set between 570 nm and 590 nm.
- Pipettes and pipette tips.
- 500 mL graduated cylinder for preparation of Wash Buffer.
- Deionized or distilled water.
- Multi-channel pipette, squirt bottle, or manifold dispenser.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 $\pm$ 50 rpm.
- 0.1 M HCl for preparation of cell lysates or tissue samples.

PRECAUTIONS

Some components of this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with the kit is a caustic material. Wear eye, hand, face, and clothing protection when using this material.

The cAMP Standard provided with this kit is supplied in ethanolic buffer at a pH optimized to maintain cAMP integrity. Care should be taken when handling this material because of the known and unknown effects of cAMP.

The activity of the cAMP Conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions and is affected by high concentrations of chelators, such as EDTA and EGTA. Samples that contain < 10 mM EDTA or EGTA can be assayed without interference. Samples that contain higher concentrations of chelators must be diluted prior to assay.

Inorganic phosphate is a strong competitive inhibitor of alkaline phosphatase; avoid the use of PBS based wash buffers and other sources of inorganic phosphate contamination.

This kits performance is tested with a variety of samples. However, it is possible that high levels of interfering substances may cause variability in assay performance.

SAMPLE COLLECTION AND STORAGE

Samples containing rabbit IgG may interfere with this assay.

Cell Culture Media - Samples in the majority of cell culture media* can be read in the assay provided the standards have been diluted into cell culture media instead of Assay Buffer ED2. There may be a small change in binding associated with running the standards and samples in media.

Cell Lysate - For adherent cell lines, remove the cell culture media* by aspirating or decanting. Add 0.1 M HCl to lyse the cells. The HCl should cover the adherent cells completely. Incubate for 10 minutes. View cells under a microscope to ensure that the cells have been lysed. If adequate lysis has not occurred, further incubate for an additional 10 minutes. Centrifuge lysates for 10 minutes at 600 x g. Supernates may be assayed directly in the low pH cAMP kit (Catalog # DE0355) or dried down prior to reconstitution in Assay Buffer ED2.

Tissue - Freeze the sample in liquid nitrogen. Keep the tissue frozen for the remainder of the preparation. Grind the frozen pellet to a fine powder using a mortar and pestle. Allow the liquid nitrogen to evaporate. Weigh the frozen tissue and homogenize in 10 volumes of cold 5% TCA in a glass-Teflon tissue grinder. Centrifuge at 600 x g for 10 minutes. Extract supernates with 3 volumes of water-saturated ether. Dry the aqueous extracts and reconstitute with Assay Buffer ED2. Run samples directly in the assay.

Saliva - Collect saliva using a Saliva Sac® or equivalent. Centrifuge at 600 x g. Prepare samples as directed in Sample Preparation and assay immediately or store at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Collect serum using a serum separator tube (SST). Centrifuge at 600 x g. Prepare samples as directed in Sample Preparation and assay immediately or store at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge at 600 x g. Prepare samples as directed in Sample Preparation and assay immediately or store at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *EDTA plasma is not recommended when running the acetylated procedure since it tends to precipitate.*

**RPMI-1640 contains high concentrations (> 350 pg/mL) of endogenous cAMP. Residual media on the cells will affect the measurement of cAMP levels.*

SAMPLE PREPARATION

All saliva, serum, and plasma samples require at least a 2-fold dilution. A suggested 2-fold dilution is 150 µL sample + 150 µL Assay Buffer ED2.

This assay is compatible with cAMP samples in a wide range of matrices. Samples diluted sufficiently into Assay Diluent ED2 (> 1:10) can be read directly from the standard curve.

ACETYLATION PROCEDURE (OPTIONAL)

If samples with very low levels of cAMP are to be measured, follow the Acetylation Procedure below.

Acetylate all Standards and samples, including the zero standard/NSB tube (Assay Buffer ED2), by adding 10 µL of the Acetylating Reagent to 200 µL Standard or sample. Add the Acetylation Reagent directly to the Standard or sample and vortex for 2 seconds. **Use the acetylated Standards and samples within 30 minutes.**

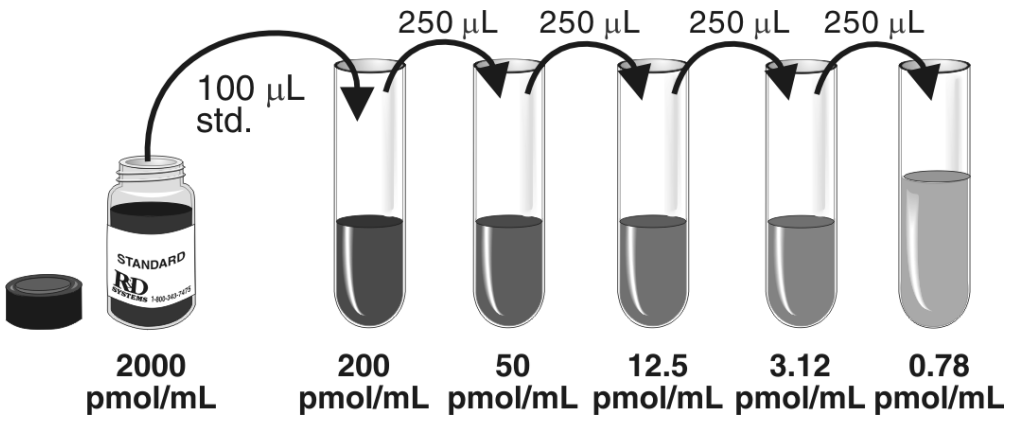
REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - Dilute 30 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 300 mL of Wash Buffer (1X).

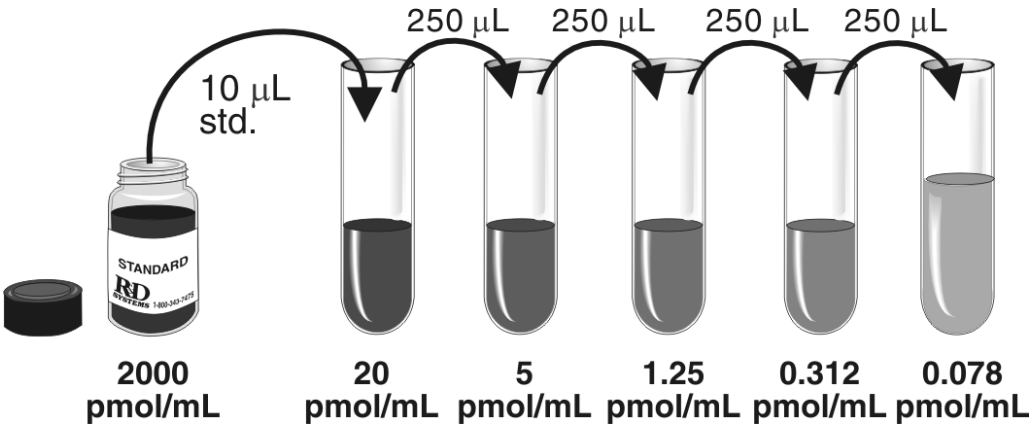
Acetylation Reagent - Add 0.5 mL of Acetic Anhydride to 1 mL of Triethylamine. Use within 60 minutes of preparation. **Caution: See Material Safety Data Sheets.**

cAMP Standard - **Non-Acetylated**
Pipette 900 µL of Assay Buffer ED2 into the 200 pmol/mL tube. Add 750 µL of Assay Buffer ED2 into the remaining tubes. *When pipetting standards, it is important to pre-rinse the pipette tip*. Use the 2000 pmol/mL standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 200 pmol/mL standard serves as the high standard and the Assay Buffer ED2 serves as the zero standard (B_0) (0 pmol/mL). **Use diluted standards within 60 minutes.**



cAMP Standard - Acetylated

Pipette 990 µL of Assay Buffer ED2 into the 20 pmol/mL tube. Pipette 750 µL of Assay Buffer ED2 into the remaining tubes. *When pipetting standards, it is important to pre-rinse the pipette tip*. Use the 2000 pmol/mL standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 20 pmol/mL standard serves as the high standard and Assay Buffer ED2 serves as the zero standard (B_0) (0 pmol/mL). **Use diluted standards within 30 minutes.**



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Total Activity (TA), Non-Specific Binding (NSB), Maximum Binding (B_0), and Substrate Blank wells should be run as a means of quality control for each assay.

If the acetylated version of the kit is to be run, acetylate all standards and samples by adding 10 µL of the Acetylating Reagent for each 200 µL of standard or sample. Add 50 µL of the Acetylating Reagent to the zero standard/NSB tube containing 1 mL of Assay Buffer ED2 and use in steps 4 and 5 below. Failure to acetylate the zero standard/NSB will result in inaccurate B/B_0 values.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Reserve wells for TA and Substrate Blank.
4. Add 150 µL of Assay Buffer to the NSB wells.
5. Add 100 µL of Assay Buffer to the zero standard (B_0) wells.
6. Add 100 µL of Standard or sample* to the remaining wells.
7. Add 50 µL of cAMP Conjugate to each well (excluding the TA and Substrate Blank wells).
8. Add 50 µL of cAMP Antibody Solution to each well (excluding the NSB, TA, and Substrate Blank wells). Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.

   Note: *The TA and Substrate Blank wells are empty at this point. The NSB wells should be blue and all other wells should be green.*
9. Aspirate or decant each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (200 µL) using a squirt bottle, multi-channel pipette, or manifold dispenser. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
10. Add 5 µL of cAMP Conjugate to the TA wells.
11. Add 200 µL of pNPP Substrate to **all wells**. Incubate for 1 hour at room temperature **on the benchtop (do not shake)**.
12. Add 50 µL of Stop Solution to each well.
13. Determine the optical density of each well **immediately**, using a microplate reader set to 405 nm with wavelength correction set between 570 nm and 590 nm.

*Samples require dilution as directed in the Sample Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and subtract the average NSB optical density.

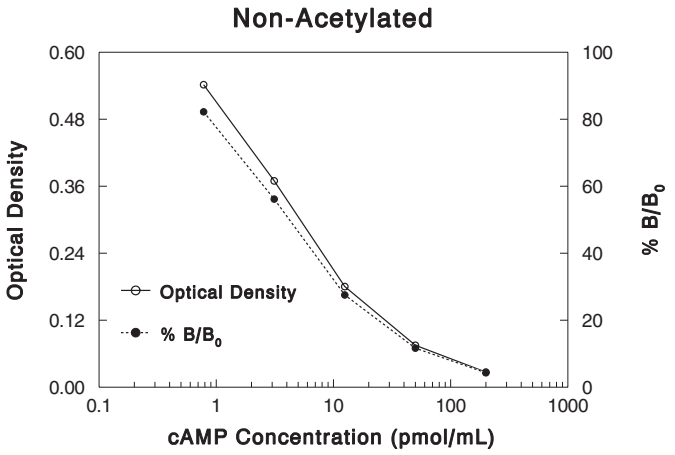
Create a standard curve by reducing the data using computer software capable of generating a four paramater logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph.

% B/B_0 can be calculated by dividing the corrected OD for each standard or sample by the corrected B_0 OD and multiplying by 100.

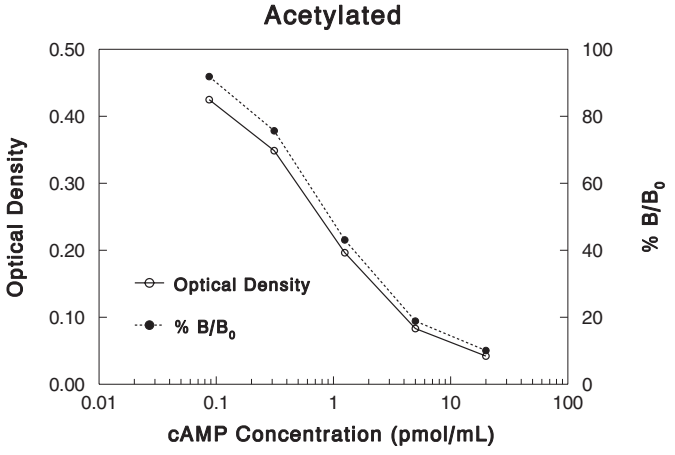
Calculate the concentration of cAMP corresponding to the mean absorbance or the % B/B_0 from the standard curve. Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.



pmol/mL	OD	Average	Corrected	% B/B_0
NSB	0.001 0.002	0.001	——	——
0 (B_0)	0.666 0.665	0.661	0.660	100
0.781	0.549 0.537	0.543	0.542	82.2
3.125	0.372 0.370	0.371	0.370	56.2
12.5	0.183 0.181	0.182	0.181	27.6
50	0.077 0.077	0.077	0.076	11.7
200	0.029 0.028	0.029	0.028	4.3



pmol/mL	OD	Average	Corrected	% B/B_0
NSB	0.004 0.003	0.004	——	——
0 (B_0)	0.465 0.468	0.467	0.463	100
0.0781	0.437 0.420	0.429	0.425	91.8
0.312	0.343 0.363	0.353	0.349	75.7
1.25	0.200 0.202	0.201	0.197	43.1
5	0.088 0.088	0.088	0.084	18.9
20	0.059 0.035	0.047	0.043	10.1

QUALITY CONTROL

Each laboratory should establish a quality control program to monitor the performace of the cAMP Immunoassay. As a part of this program, TA, NSB, B_0, and Substrate Blank should be run in each assay.

Typical Quality Control Parameters

	Non-Actylated	Acetylated
Substrate Blank (O.D.)	0.076	0.078
Total Activity (TA x 10)	15.65	17.92
% NSB (NSB/TA x 100)	0.006%	0.02%
% B_0 (B_0/TA x 100)	4.2%	2.6%
Quality of Fit	0.999	0.999

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty-four times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in eight separate assays to assess inter-assay precision.

	Non-Acetylated						Acetylated					
	Intra-assay			Inter-assay			Intra-assay			Inter-assay		
Sample	1	2	3	1	2	3	1	2	3	1	2	3
n	24	24	24	8	8	8	24	24	24	8	8	8
Mean (pmol/mL)	1.18	5.96	18.6	1.13	4.95	19.2	0.40	0.90	5.58	0.46	0.98	4.75
Standard Deviation	0.12	0.15	0.54	0.15	0.55	1.61	0.03	0.06	0.43	0.05	0.11	0.38
CV (%)	10.5	2.5	2.9	13.7	11.2	8.4	7.4	6.8	7.7	11.2	11.2	7.9

RECOVERY

The recovery of cAMP spiked into samples in various matrices was evaluated.

	% Recovery	
Sample Type	Non-Acetylated	Acetylated
Porcine Serum*	103%	96%
Human Saliva*	95%	96%

*Samples were diluted prior to assay as directed in Sample Preparation.

LINEARITY

To assess the linearity of the assay, Assay Buffer ED2 containing cAMP was assayed using serial 2-fold dilutions.

Non-Acetylated				Acetylated			
Dilution	Observed (pmol/mL)	Expected (pmol/mL)	% Observed / Expected	Dilution	Observed (pmol/mL)	Expected (pmol/mL)	% Observed / Expected
Neat	49.2	—	—	Neat	5.42	—	—
1:2	23.1	24.6	94	1:2	2.86	2.71	106
1:4	13.7	12.3	112	1:4	1.23	1.36	91
1:8	6.9	6.15	112	1:8	0.51	0.68	75
1:16	3.41	3.07	111	1:16	0.28	0.34	83

SENSITIVITY

The sensitivity of the cAMP assay is typically less than 0.30 pmol/mL for the non-acetylated procedure and 0.039 pmol/mL for the acetylated procedure.

Sensitivity was determined by subtracting two standard deviations from the mean optical density value of sixteen zero standard (B_0) replicates and calculating the corresponding concentration.

CROSS-REACTIVITY

Cross-reactivity for the following compounds was determined by adding the cross-reactant to Assay Buffer ED2 at concentrations from 2 pmol/mL to 2000 pmol/mL. The cross-reactivity was calculated at 50% B/B_0. No cross-reactivity was observed.

AMP	cGMP	GTP	CTP
ATP	GMP	cUMP	

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